Protocols for Team UCC Ireland

Protocol for splitting cells (RAW 264.7 macrophages, adherent murine cell line)

Cells are to be split when cells have reached 80% confluence.

Reagents required: phosphate buffer saline (PBS), trypsin, Dulbecco's Modified Eagle's Medium (DMEM),

Procedure:

1. Discard media without touching cells.
2. Wash twice with PBS.
3. Add 2mL of trypsin - shake to evenly distribute.
4. Incubate for 3 minutes at 37℃ 5% CO_2.
5. Check in the microscope for non-adherence/released cells.
6. If cells are still adhering, scrape the cells using a cell scraper.
7. Deactivate trypsin by adding 8mL DMEM - shake to evenly distribute.
8. Extract the cells and media - transfer to 15mL falcon centrifuge tube.
9. Centrifuge at 1000rpm for 5 minutes.
10. Label T175 flasks and add 18mL of DMEM to flasks.
11. Remove solution by inversion.
12. Resuspend pellet in 10mL DMEM.
13. Mix with pipette.
14. Extract 2mL of cells in DMEM and add into T175 flasks.
15. Incubate cells at 37℃ 5% CO_2.
16. Clean the working area, discard material and wah waste bin.

Lactococcus lactis Transformation via electroporation Protocol

1. Grow lactococcal strain o/vernight at 30°C in GM17 broth (40mL culture).
2. Inoculate 40mL (10% inoculum) into 400mL SGM17 broth with 20Ml of 10% glucose.
3. Incubate at 30°C for 5 hours.
4. Transfer to cold sterile centrifuge (Sorvall) bottles.
5. Harvest cells by centrifugation (cold rotor 4°C) at 4,500rpm for 10 minutes.
6. Wash cells twice with 25mL ice cold 0.5M sucrose 10% glycerol solution.
7. Resuspend cells in 1mL ice cold buffer (contains??5mM glucerol buff), aliquot and store at -80°C.
8. Electrotransform cells under following conditions: 25mF, 200Ω, 2kV, 50µL cells and 5µL dialysed DNA in cold cuvettes (include negative control with no DNA).

9. After electroporation, resuspend cells in GM17 broth (950µL) and incubate at 30°C for 2 hours.
10. Plate dilutions on GM17 agar and incubate anaerobically/aerobically for 24-48 hours.

Transformation via heat shock

1. Take competent cells out of -80°C and thaw on ice.
2. Take agar plates (containing the appropriate antibiotic) out of 4°C to warm up to room temperature or place in 37°C incubator.
3. Mix 1 to 5µl of DNA (usually 10pg to 100ng) into 50µL of competent cells in a microcentrifuge. GENTLY mix by flicking the bottom of the tube with your finger a few times.
4. Place the competent cell/DNA mixture on ice for 25 mins
5. Heat shock each transformation tube by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 seconds.
6. Put the tubes back on ice for 5 min.
7. Add 1000µl LB or SOC media (without antibiotic) and grow in 37°C shaking incubator for 45min.
8. Plate some or all of the transformation (100 ul) onto a 10cm LB agar plate containing the appropriate antibiotic.
9. Incubate plates at 37°C overnight.

Pouring Agar Plates

1. Weigh out the following into a 1L Erlenmeyer flask:

- 5g NaCl
- 5g Tryptone
- 2.5g Yeast Extract
- 7.5g Agar
- add dH2O to 500mL

2. Swirl to mix - the contents do not have to be completely in solution, but any powder left on the sides of the flask will caramelize on the glass during autoclaving.

3. Cover the top of the flask with aluminium foil and label with autoclave tape.

4. Autoclave on the liquid setting for 20 minutes.

5. After removing the solution from the autoclave, allow the agar solution to cool to 55°C.Place in water bath at 55 degrees to do this.

6. When pouring plates, keep your bench area sterile by working near a flame or bunsen burner. Also clean it down with ethanol prior.

7. Add the appropriate amount of desired antibiotic to the solution.

8. Pour ~20mL of LB agar per 10cm polystyrene Petri dish.

9. Place the lids on the plates and allow them to cool for 30-60 minutes (until solidified), then invert the plates. Let sit for several more hours or overnight.

10. Label the bottom of plates with antibiotic and date and store in plastic bags or sealed with parafilm at 4°C.

Ligations

1. Combine the following in a PCR or Eppendorf tube:
- 25ng Vector DNA
- 75ng Insert DNA (maintain 3:1)
- Ligase Buffer (1µL/10µL reaction for 10X buffer, and 2µL/10µL reaction for 5X buffer)
- 1µL T4 DNA Ligase
- H20 to a total of 10µL

2. Incubate at room temperature for 2hr, or at 16°C overnight depending on the nature of the DNA being ligated.
3. Heat shock at 65℃ for 10 minutes
4. Proceed with bacterial transformation

MULTI-STEP ASSEMBLY USING GIBSON ASSEMBLY

The 4 DNA sequences to be ligated were as follows: pNZ44 (vector), 3394bp, Gibson fragment 1 (RBS, USP45 secretion signal, His tag) - 173bp, Gibson fragment 2 (dCas9 amplified from MSP712 plasmid) - 4728bp, Gibson fragment 3 (KRAB domain, IKK2 sgRNA).

1. Assembly of pNZ44 and Gibson fragment 1--->A. 1:5 ratio (excess of GF1)

NEB 2x HiFi DNA Assembly Master Mix	10uL
pNZ44 (117ng/uL)	1.8uL
Gibson fragment 1(11ng/uL)	4.85uL
dH2O	3.35uL
Total	20uL

Incubated at 50 degrees for 1 hour

2. Assembly of GF2 (4150bp) and GF3 (573bp) --->B Twofold excess of GF3 (smaller fragment). 0.1pmol of GF2, 0.2pmol of GF3:

NEB 2x HiFi DNA Assembly Master Mix	10uL
GF2 (93.7ng/uL)	2.74uL

GF3 (11.7ng/uL)	6.05uL
dH2O	1.21uL
Total	20uL

3. Final assembly of A and B. Took two approaches to this:

i) Took 5uL of both A and B. Added 10uL of NEB 2x HiFi DNA Assembly Master Mix. Incubated at 50 degrees for 1 hour.
ii) Took 10uL of both A and B. Didn't add NEB 2x HiFi DNA Assembly Master Mix. Incubated at 50 degrees for 1 hour.

find manufacturer of M17 for reference. Can also use https://www.bd.com/europe/regulatory/Assets/IFU/Difco_BBL/218561.pdf for reference for 11g/L agar)